Identification of Potential Biofertilizer and Bioremediator Bacteria from Upland Soil Based on 16s rDNA Sequence Analysis

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ABSTRACT

The long-term presence of synthetic pesticides on agricultural land can lead to a decline in soil fertility. Synthetic pesticides inhibit the activity of essential enzymes in the soil and suppress beneficial microbial populations for plants. One potential approach to mitigate the extent of contamination caused by synthetic pesticides involves the utilization of indigenous pesticide-resistant bacteria. Several upland soil bacteria from Banyumas Regency, Central Java Province, Indonesia, were successfully isolated from a previous study. The isolated bacteria have the potential to be developed as pesticide bio-remediators and biofertilizers. The bacterial isolates are expected to have characters that support plant growth through their ability to provide dissolved phosphate. However, the potential bacterial isolates need to be identified by molecular approaches. This study was conducted to identify bacterial isolates of GT2, SR1, SW1, and PA1 by 16S rDNA sequencing analysis. The results showed that isolate GT2 was placed within a group of reference strains of *Bacillus proteolyticus*, isolate SR1 was placed within a group of *B. paramycoides*, isolate SW1 was set within a group of *B. albus*, and isolate PA1 was placed within a group of *Acidovorax delafieldii*. The genetic distance of isolate GT2 and *B. B. proteolyticus,* isolate SR1 and *B. paramycoides,* isolate SW1 and *B. albus* were 0.0000 each, and isolate PA1 and *A. delafieldii* were 0.0061.

Keywords: Biofertilizer; Bio-remediator; Upland soil bacteria

ABSTRAK

Kontaminasi pestisida sintetik pada lahan pertanian dalam waktu lama dapat menyebabkan penurunan kesuburan tanah. Pestisida sintetik menghambat aktivitas enzim-enzim penting di dalam tanah dan menekan populasi mikroba yang bermanfaat bagi tanaman. Untuk mengurangi tingkat pencemaran pestisida sintetik dapat digunakan bakteri lokal yang resisten terhadap pestisida. Sejumlah bakteri tanah lahan marginal (kering-masam) dari Kabupaten Banyumas, Provinsi Jawa Tengah, Indonesia berhasil diisolasi. Isolat bakteri tersebut berpotensi dikembangkan sebagai bioremediator pestisida. Isolat bakteri juga diketahui memiliki karakter yang mendukung pertumbuhan tanaman melalui aktivitasnya melarutkan fosfat sehingga tersedia bagi tanaman. Isolat bakteri tersebut berpotensi dikembangkan sebagai pupuk hayati. Isolat bakteri potensial perlu diidentifikasi dengan pendekatan molekuler. Penelitian ini bertujuan untuk mengungkap identitas isolat bakteri GT2, SR1, SW1, dan PA1 dengan analisis sekuensing 16S rDNA. Hasil penelitian menunjukkan bahwa isolat GT2 ditempatkan dalam kelompok strain referensi Bacillus proteolyticus, isolat SR1 ditempatkan dalam kelompok B. paramycoides, isolat SW1 ditempatkan dalam kelompok B. albus, dan isolat PA1 ditempatkan dalam kelompok Acidovorax delafieldii. Jarak genetik isolat GT2 dan B. proteolyticus, isolat SR1 dan B. paramycoides, isolat SW1 dan B. albus masing-masing adalah 0,0000, dan isolat PA1 dan A. delafieldii adalah 0,0061.

Kata kunci: *Biofertilizer; Bioremediator; Bakteri lahan marginal*

INTRODUCTION

Contamination of synthetic pesticides on agricultural land is known to reduce soil fertility. Synthetic pesticides, such as buprofezin, are generally used to control planthopper pests in rice cultivation in Indonesia. Unfortunately, buprofezin accumulation in the soil for a long time is known to cause a decrease in agricultural land fertility. Buprofezin can interfere with soil enzyme activity, such as invertase ([Maddela & Venkateswarlu, 2018a](#)), amylase ([Maddela & Venkateswarlu, 2018b](#)), phos-

phatase (Maddela & Venkateswarlu, 2018c), and urease (Maddela & Venkateswarlu, 2018d). These enzymes play a role in providing nutrients plants need, including phosphate, nitrogen, and others. The availability of nutrients is essential for the level of soil fertility. Phosphate is necessary for energy transport, cellular structures, and nucleic acids and is thus essential for life (Margalef et al., 2017). Organic nitrogen from soil enzyme mineralization supports plant growth and yield (Grzyb et al., 2021). Buprofezin also suppresses the population of soil microorganisms that are beneficial to plants. Therefore, some countries prohibit the use of this type of pesticide to control plant pests (Qureshi et al., 2016).

To increase the fertility of agricultural soil, the use of pesticide-resistant bacteria, which can reduce the level of pesticide contamination (acting as a bio-remediator) and support plant growth (working as a biofertilizer), is needed in the stages of plant cultivation. Several soil bacteria from upland rice fields in Central Java Province, Indonesia, were successfully isolated from our previous study (Hadi et al., 2019). The four isolates of these dominant bacterial isolates (code: GT2, SR1, SW1, and PA1) are known to be resistant to buprofezin (one of the pesticide-active ingredients used to control planthopper pests in rice cultivation). Using local bacterial isolates to overcome the problem of buprofezin residue contamination is an essential part of efforts to rehabilitate agricultural lands contaminated with pesticide residues to support sustainable agricultural efforts. The bacterial isolates also have other potential to support direct plant growth through their ability to provide dissolved phosphate (Hadi et al., 2021). Phosphate solubilizing bacteria (PSB) support plant growth, especially in land where phosphate availability is poor, such as ultisol soil.

The morphological and biochemical character-ization showed that GT2, SR1, SW1, and PA1 were Bacillus sp (Hadi et al., 2019). Identifying potential dominant bacterial isolates at the species level is needed, especially for further developing bacterial isolates as biofertilizers and pesticide bioremediation agents. The future industrial application of the potential bacterial isolates will be possible after the biological material's characterization, identification, and taxonomic classification (Franco-Duarte et al., 2019). The title of bacterial isolates to determine the species can be done using a molecular approach.

The identification of bacterial species by molecular-based analysis commonly involves the utilization of 16S ribosomal RNA gene sequences (16S rDNA). Compared to phenotypic methods (morphological and biochemical characteristics), identification of bacteria by 16S rDNA gene sequencing is considered more accurate (Nurhayati, 2018). Molecular-based analysis is an efficient, proper, cheap, and standardized method for identifying organisms (Lebonah et al., 2014). PCR-based identification of bacterial DNA through sequencing of the 16S rDNA gene has become a standard molecular method. PCR-based methods are faster than conventional culture-based methods but are also helpful in identifying bacteria that are difficult to grow in laboratory conditions (Franco-Duarte et al., 2019).

The 16S rDNA gene sequences have conserved areas, so they are very suitable for sequencing techniques to identify a species, genetic diversity, and relationships. The 16S rDNA gene is approximately 1500 bp long, consisting of nine (hyper)variable regions named V1 to V9, interspaced with more conserved regions (Winand et al., 2020). This area is specific to organisms, which distinguish between species. Generally, this area is targeted for sequencing. Oligonucleotide sequencing is designed to amplify (multiply) this region's sustainable and

hypervariable regions. Eventually, you will get a unique sequence for each organism, distinguishing it from other organisms. The objectives of this study were to reveal the identity of bacterial isolate GT2, SR1, SW1, and PA1 by 16S rDNA sequencing analysis.

MATERIALS AND METHODS

Bacterial Isolates

Four bacterial isolates showing various potential were obtained from the previous study (Table 1). Those isolates were already preserved in the Laboratory of Agroecology, Faculty of Agriculture, Universitas Jenderal Soedirman, Purwokerto, Central Java Province, Indonesia.

DNA Genome Isolation and 16S rDNA Amplification

Pure and single bacterial isolates were prepared for DNA genome isolation using the Quick-DNA ™ Fungal / Bacterial Miniprep Kit method from

Table 1. The potential characteristics of the bacterial isolate in this study

Code	Source	Potential characteristics
GT2	Gunungtugel Village	Phosphate solubilizer, Buprofezine resistance and bio-remediator
SR1	Srowot Village	Phosphate solubilizer, Starch hydrolysis, Buprofezine resistance and bio-remediator
SW1	Sokawera Village	Starch hydrolysis, Buprofezine resistance and bio-remediator
PA1	Pagaralang Village	Phosphate solubilizer, Buprofezine resistance and bio-remediator

Zymo Research. The concentration and purity of isolated genomic DNA were measured by nano-drop spectrophotometer at 260 and 280 nm, and 16S rDNA amplification was performed using Polymerase Chain Reaction (PCR).

PCR amplification of 16S rDNA gene of GT2 and SR1 referred to MyTaq HS Red Mix method (Bioline, BIO-25047): 9 μL ddH$_2$O, 12.5 μL 2x MyTaq Red Mix, 1.0 μL 20 μmol/μl 27F primer, 1.0 μL 20 μmol/μl 1492R primer, and 1 μL DNA template. PCR amplification of 16S rDNA gene of SW1 and PA1 referred to KOD FX Neo method (Toyobo, KFX – 201): 5 L ddH$_2$O, 10.5 μL 2x PCR buffer for KOD FX Neo, 5 μL 2mM dNTPs, 0.5 L 10 pmol/μl (μM) 27F, 0.5 μL 10 pmol/μl (μM) 1492R primer, 0.5 L 1.0 U/ μl KOD FX Neo, and 3 L DNA template. PCR condition for GT2 and SR1: Initial denaturation (95ºC, 60 seconds), denaturation (95ºC, 15 seconds), annealing (52ºC, 15 seconds), extension (72ºC, 45 seconds), and hold (4ºC ∞). Amplification was carried out for 35 cycles. PCR condition for SW1 and PA1: Initial denaturation (98ºC, 180 seconds), denaturation (98ºC, 15 seconds), annealing (52ºC, 30 seconds), extension (68ºC, 45 seconds), post-extension (68ºC, 180 seconds), and hold (4ºC, ∞). Amplification was carried out for 35 cycles. Amplification of the 16S rDNA sequence using a labcycler machine from Sensoquest GmbH, Germany.

The PCR product was examined by agarose gel electrophoresis technique to estimate the length of a 16S rDNA gene. The voltage used was 100 volts. DNA bands were visualized under ultraviolet (UV) lights at 300 nm.

16S rDNA Sequencing, Phylogenetic Trees Reconstruction, and Genetic Distance Analysis

PCR products were purified by Zymoclean ™ Gel DNA Recovery Kit. The 16S rDNA gene was sequenced using the BigDye®Terminator v3.1 Cycle Sequencing Kit by Applied Biosystem method. Sequencing analysis was performed using 27F (AGAGTTTGATCMTGGCTCAG), 785F (GGATTAGATACCCTGGTA), and 1492R (TAC-

GGYTACCTTGTTACGACTT) oligonucleotide primer. PT Genetika Science Indonesia conducted the sequencing analysis of 16S rDNA.

The data from the sequencing analysis were used for bioinformatics analysis using the N-Blast program, which can be accessed on the website https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome. After obtaining the results, ten comparative bacterial species sequences with the highest percentage similarity were downloaded and stored in fasta format. The whole sequence (4 samples of GT2, SR1, SW1, PA1, and 10 comparison sequences) was aligned by MEGA version X (Kumar et al., 2018). The alignment result was also analyzed using MEGA version X by the Maximum Likelihood method to assemble the phylogenetic tree and to evaluate the genetic relationships of GT2, SR1, SW1, and PA1 isolates.

RESULTS AND DISCUSSION
DNA Genome Isolation

The nucleotide sequences of the 16S rDNA sequences were acquired by isolating the genomic DNA of the bacterial isolates GT2, SR1, SW1, and PA1. The concentration and purity level of genomic DNA of bacterial isolates obtained from the results of spectrophotometer analysis are presented in Table 2.

Based on the data presented in Table 2, the highest concentration of genomic DNA was found in isolates GT2, which was 361.4 ng/µl, while the lowest was in isolates PA1, which was 79.8 ng/µl. All isolated genomic DNA can be used to amplify the 16S rDNA sequence using the PCR technique. DNA concentrations commonly used in PCR techniques range from 25-500 ng/µl (Hidayati et al., 2016). The accuracy of the DNA concentration also determines the success of the target region amplifi-

**Table 2.** Concentration and purity level of isolated genome DNA

No	Code	Concentration (ng/µl)	Absorbance A260/280 (purity)
1	GT2	361.4	1.87
2	SR1	175.9	1.88
3	SW1	90.1	1.72
4	PA1	79.8	1.85

cation process in genomic DNA through the PCR technique. For the purity level of isolated DNA, all isolates were in the range of 1.8-2.0 (except isolate SW1). The 260/280 nm ratio of 1.8 indicated that the isolated DNA had high purity without proteins and phenols (Abdel-latif & Osman, 2017). DNA with an A260/A280 ratio value of less than 1.8 indicates the presence of phenolic contaminants or other compounds.

The 16S rDNA Amplification

Genomic DNA in sufficient quantity and purity was used as a template to amplify 16S rDNA sequences for sequencing needs.

Based on the amplification result in Figure 1, the 16S rDNA gene sequences of four different bacterial isolates were successfully reproduced. The amplification quality of the 16S rDNA gene
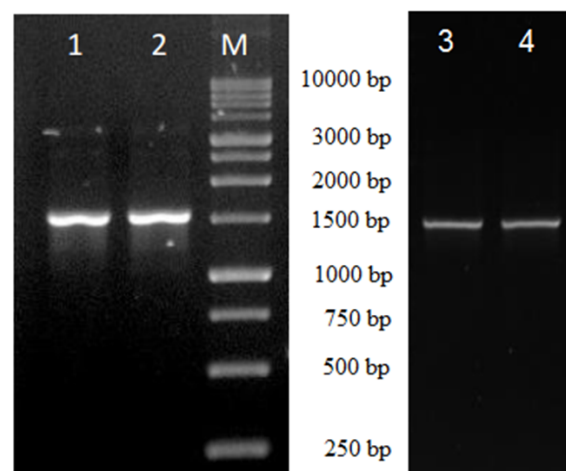
Figure 1. PCR product of DNA visualization with 1% agarose gel electrophoresis
1: GT2, 2: SR1, M: 1 kb DNA *ladder,* 3: SW1, 4: PA1; The 16S rDNA gene sequence measuring about 1500 bp in length was successfully amplified by PCR technique.

Table 3. N-Blast result of 16S rDNA sequence of bacterial isolates

| No | Code | N-Blast | | | | | | |
		Max Score	Total Score	Query Coverage	E Value	Percentage Identity	Similar species	Accession
1	GT2	2627	2627	100%	0.0	100%	*Bacillus proteolyticus* strain MCCC 1A00365	NR_157735.1
2	SR1	1910	1910	100%	0.0	100%	*B. paramycoides* strain MCCC 1A04098	NR_157734.1
3	SW1	2586	2586	98%	0.0	100%	*B. albus* strain MCCC 1A02146	NR_157729.1
4	PA1	2525	2525	98%	0.0	99.42%	*Acidovorax delafieldii* strain *133*	NR_028714.1

sequences of isolates SW1 and PA1 was better than that of GT2 and SR1 isolates (Figure 1). The indication is the appearance of smears (shadows around the target DNA band) on the 16S rDNA sequences of GT2 and SR1 isolates. According to Zrimec et al. (2013), band smearing occurs due to imperfectly paired strands of the amplified DNA. Thus, the smear that appears on the amplification results of the 16S rDNA sequence does not affect the quality of the sequencing analysis, and the 16S rDNA sequence needs to be purified. The impurity of target DNA as a template source in sequencing analysis can lead to inaccurate interpretation of the DNA.

The 16S rDNA Sequencing Analysis

The 16S rDNA gene sequences of bacterial isolates GT2, SR1, SW1, and PA1 were known based on sequencing analysis using oligonucleotide primers 27F, 785F, and 1492R. The results of data processing using Bioedit software showed that the consensus sequences length from 16S rDNA sequencing was 1422 bp (isolate GT2), 1034 bp (isolate SR1), 1400 bp (isolate SW1), and 1391 bp (isolate PA1). The 16S rDNA gene sequences of the bacterial isolates have been uploaded to the GenBank website with accession numbers MN788651 for GT2, MN788649 for SR1, MN788652 for SW1, and MN788650 for PA1. The nucleotide sequence obtained from the sequencing was used

to determine the species identity of the target bacterial isolate. The method used was N-blast analysis. N-Blast analysis was used to assess the similarity of the 16S rDNA sequences of GT2, SR1, SW1, and PA1 with bacterial isolates in the NCBI database. The results of the N-Blast analysis of 16S rDNA sequences are presented in Table 3.

Based on Table 3, the bacterial isolate GT2 was suspected to be a species of B. proteolyticus. The percentage identity value between isolate GT2 and B. proteolyticus strain MCCC 1A00365 (Acession NR_157735.1) were 100%. The bacterial isolate SR1 was suspected to be a B. paramycoides species because the percentage identity value reached 100% compared to B. paramycoides strain MCCC 1A04098 (Acession NR_157734.1). Based on the percentage identity value, the bacterial isolate SW1 was suspected to be a B. albus species, which reached 100% compared to B. albus strain MCCC 1A02146 (NR_157729.1). Meanwhile, the PA1 bacterial isolate with the lowest percentage identity compared to the three other sample bacterial isolates had a percentage identity value of 99.4% compared to Acidovorax delafieldii strain 133 (NR_028714.1). A sample can be considered identical species if the percentage identity value is more significant than 97.5% (Stackerbrandt & Goebel, 1994). Referring to Drancourt et al. (2000), the species level of bacterial isolates is achieved when the 16S rDNA sequence similarity is at least 99%,
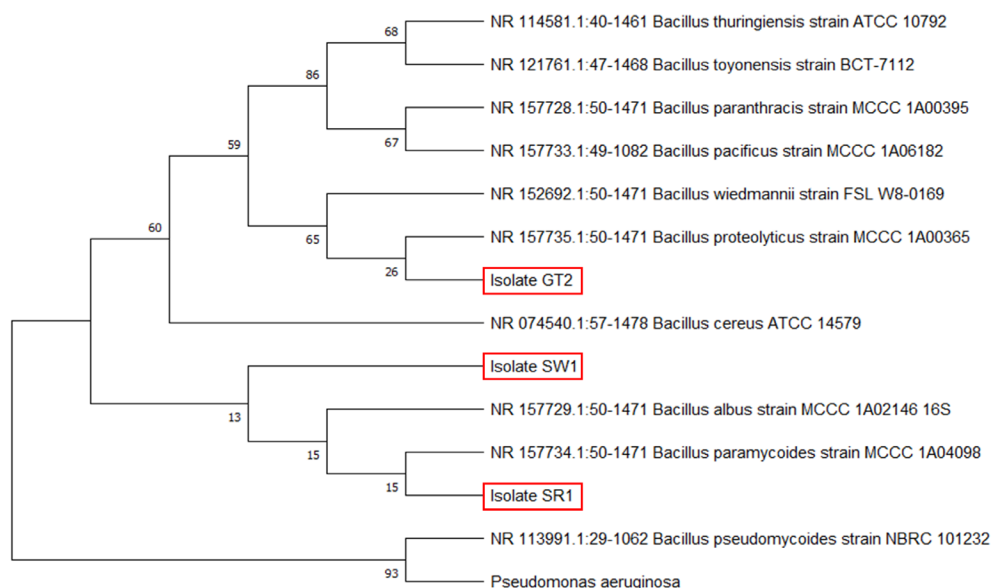
Figure 2. Phylogenetic tree of isolates GT2, SW1, SR1, and several similar bacterial isolates from NCBI database. The trees were constructed by using the Maximum Likelihood method and Tamura-Nei model with 1000x bootstrap analysis
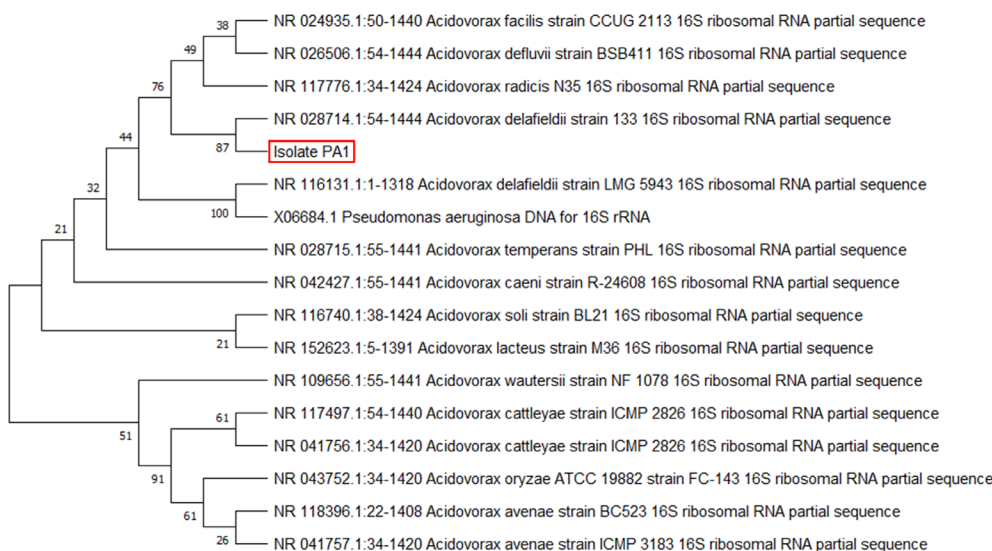
Figure 3. Phylogenetic tree of isolates PA1 and several similar bacterial isolates from NCBI database. The trees were constructed by using the Maximum Likelihood method and Tamura-Nei model with 1000x bootstrap analysis.

and the genus level is achieved when the similarity value is 97%. The results of the N-Blast analysis in Table 3 are supported by data on the potential characteristics of bacteria in which Bacillus species generally can dissolve phosphate (Saeid et al., 2018). Bacillus species also resist synthetic pesticides and can break them down into harmless compounds (Kumar et al., 2019).

Phylogenetic Tree Analysis

Ten similar 16S rDNA sequences from N-Blast were downloaded and used for phylogenetic tree reconstruction. The method for assembling phylo-

genetic trees was the Construct / Test Maximum Likelihood Tree of the MEGA version X. The phylogenetic trees of GT2, SR1, SW1, and PA1 isolates were presented in Figures 2 and 3.

GT2 isolates were in a sub-cluster with B. Proteolyticus, SW1 isolates were in a sub-cluster with B. albus, SR1 isolates were in a sub-cluster with B. paramycoides (Figure 2), and isolate PA1 was in a sub-cluster with A. Delafieldii (Figure 3). These data support the previous N-Blast result (Table 3). In this study, P. aeruginosa was used as an assembly outgroup species in a phylogenetic tree, such as the launch of Osman & Yin (2018). Outgroup species were used for comparison with in-group species.

The results showed the genetic distance of the sample bacterial isolates compared to several bacteria from NCBI database. For example, the bacterial isolate GT2 had the closest genetic distance of 0.0000 to B. proteolyticus, the bacterial isolate SR1 had the most relative genetic distance of 0.0000 to B. albus and B. paramycoides, and the bacterial isolate SW1 had the closest genetic distance of 0.0000 to B. albus compared to other species in the Bacillus group. These genetic distance data support the results of the phylogenetic tree reconstruction showing that the GT2 bacterial isolates were included in the sub-group with B. proteolyticus, the SR1 bacterial isolates were included in the sub-group with B. paramycoides, and the SW1 bacterial isolates were included in the B. albus sub-group. Meanwhile, the PA1 bacterial isolate had the closest genetic distance of 0.0061 to Acidovorax delafieldii compared to the Bacillus group, indicated by the results of biochemical tests. This difference in results is possible because some morphological and biochemical test results of several bacteria sometimes show similar results, making it difficult to distinguish.

CONCLUSIONS

The result showed that isolate GT2 was placed within a group of reference strains of *Bacillus proteolyticus*, isolate SR1 was placed within a group of *B. paramycoides*, isolate SW1 was set within a group of *B. albus*, and isolate PA1 was placed within a group of *Acidovorax delafieldii*. **The genetic distance of isolate GT2 and *B. proteolyticus* was 0.0000, isolate SR1 and *B. paramycoides* was 0.0000, isolate SW1 and *B. albus* was 0.0000, and isolate PA1 and *A. delafieldii* was 0.0061.**

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